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Amy L. Strong,[1] Zhenzhen Shi,[1] Michael J. Strong,[2,3] David F.B. Miller,[4,5] Douglas B. Rusch,[6] Aaron M. Buechlein,[6] Erik K. Flemington,[2,3] John A. McLachlan,[7] Kenneth P. Nephew,[4] Matthew E. Burow,[3,8] and Bruce A. Bunnell[1,7]

[1]Center for Stem Cell Research and Regenerative Medicine, Tulane University School of Medicine, New Orleans, Louisiana, USA; [2]Department of Pathology, Tulane University School of Medicine, New Orleans, Louisiana, USA; [3]Tulane Cancer Center, Tulane Health Sciences Center, New Orleans, Louisiana, USA; [4]Medical Sciences, Indiana University, School of Medicine, Bloomington, Indiana, USA; [5]Department of Cellular and Integrative Physiology, Indiana University, School of Medicine, Bloomington, Indiana, USA; [6]Center for Genomics and Bioinformatics, Indiana University, Bloomington, Indiana, USA; [7]Department of Pharmacology, Tulane University School of Medicine, New Orleans, Louisiana, USA; [8]Department of Medicine, Section of Hematology and Medical Oncology, Tulane Health Sciences Center, New Orleans, Louisiana, USA

Address correspondence to Bruce A. Bunnell, Center for Stem Cell Research and Regenerative Medicine, Tulane University, School of Medicine, 1430 Tulane Avenue, SL-99, New Orleans, Louisiana 70112, USA. Telephone: (504) 988-7711. E-mail: bbunnell@tulane.edu. Matthew E. Burow, Department of Medicine, Section of Hematology and Medical Oncology, Tulane Health Sciences Center, 1430 Tulane Avenue, SL-78, New Orleans, Louisiana 70112. Telephone: (504) 988-6688. E-mail: mburow@tulane.edu

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Abstract

Background: Although the global use of the endocrine disrupting chemical DDT has decreased, its persistence in the environment has resulted in continued human exposure. Accumulating evidence suggests that DDT exposure has long-term adverse effects on development, yet the impact on growth and differentiation of adult stem cells remains unclear.

Objectives: Human mesenchymal stem cells (MSCs) exposed to DDT were used to evaluate the impact on stem cell biology.

Methods: DDT-treated MSCs were assessed for self-renewal, proliferation, and differentiation potential. Whole genome RNA-sequencing was performed to assess gene expression in DDT-treated MSCs.

Results: MSCs exposed to DDT formed fewer colonies, suggesting a reduction in self-renewal potential. DDT enhanced both the adipogenic and osteogenic differentiation, which was confirmed by increased mRNA expression of glucose transporter type 4 (*GLUT4*), lipoprotein lipase (*LpL*), peroxisome proliferator-activated receptor (*PPAR*)-gamma, leptin, osteonectin, core binding factor 1 (*CBFA1*), and FBJ murine osteosarcoma viral oncogene homolog (*c-Fos*). Expression of factors in DDT-treated cells was similar to estrogen-treated MSCs, suggesting that DDT may function via the estrogen receptor (ER)-mediated pathway. The co-administration of ICI 182,780 blocked the effects of DDT. RNA sequencing revealed 121 genes and non-coding RNAs to be differentially expressed in DDT-treated MSCs compared to controls cells.

Conclusion: Human MSCs provide a powerful biological system to investigate and identify the molecular mechanisms underlying the effects of environmental agents on stem cells and human health. MSCs exposed to DDT demonstrated profound alterations in self-renewal, proliferation,

differentiation, and gene expression, which may partially explain the homeostatic imbalance and increased cancer incidence among those exposed to long-term EDCs.

Introduction

Endocrine disrupting chemicals (EDCs) are natural or synthetic compounds capable of altering hormonal and homeostatic systems. While factors such as age, duration, EDC type, and dose-response dynamics influence the severity of EDC-driven changes, these chemicals remain a cause for concern due to their similarity to endogenous hormones and their induction of downstream signaling cascades (Diamanti-Kandarakis et al. 2009). Evidence suggests that epigenetic modifications associated with EDC exposure may play a role in adverse outcomes both early in development and within stem cells in adult tissues (Crews and McLachlan 2006). Thus, mechanistic studies that explore the impact of EDCs on stem cell biology and fate are critical.

DDT is an EDC, whose effects on estrogen receptor (ER) function are well documented. DDT is a widespread environmental contaminant due to its prevalent use in both agriculture and malaria control. DDT is known to negatively influence reproductive development via disruption of multiple endocrine pathways (Holm et al. 2006). Studies have demonstrated that DDT disrupts both male and female reproductive organs. Female rats exposed to DDT induced high levels of gonadotropin-releasing hormone, resulting in premature development of the reproductive system (Gregorio et al. 2001; Rasier et al. 2007; Rasier et al. 2008).

DDT levels in breast milk have been reported to exceed the tolerable daily intake and maximum residue limit in dairy milk consumed by adults, thereby increasing the exposure of infants to DDT through breast feeding (Bouwman et al. 2006; Okonkwo et al. 2008). In developing countries, rates of urogenital malformations in male infants were elevated among those exposed to DDT (Bornman et al. 2010). The continuous exposure to DDT throughout childhood and adolescence results in significant reproductive abnormalities, germ line cancers, sexual

precocity, early puberty, and reduced pregnancy rates in females (Cohn et al. 2003; Krstevska-Konstantinova et al. 2001). Several studies indicated an inverse correlation between DDT and blood hormone levels, resulting in low infant birth weight, cryptorchidism, decreased sperm motility and semen quality in males (Bhatia et al. 2005; Longnecker et al. 2002; Rhouma et al. 2001). While the precise mechanism by which DDT influences germ line cells is unknown, DDT and related organochlorine pesticides have long been know to mimic estrogens (Bulger and Kupfer 1983). Recently, DDT has been shown to exert estrogenic effects through a variety of molecular mechanisms involving ER-alpha (ERα), and other cellular signaling systems such as AP-1, p38, and ERK1/2 (Bratton et al. 2012; Frigo et al. 2005; Han et al. 2008; McLachlan et al. 2012). Although it has been established that DDT exposure alters both cellular and molecular phenotypes in humans strikingly little is understood about its effects on tissue resident stem cells. Mesenchymal stem cells (MSCs) in the bone marrow are directly involved in hematopoietic stem cell maturation and migration into the circulation, and they are key regulators of the immune system (Auletta et al. 2012). Due to their immunomodulatory and regenerative potential, MSCs are currently being investigated and used clinically to treat autoimmune diseases and regenerate bone and cartilage (Granero-Molto et al. 2008; Yamout et al. 2010). MSCs undergo self-renewal and can be efficiently differentiated into bone, adipose, and cartilage tissue. Understanding the impact of EDCs on the efficiency of MSCs self-renewal, proliferation, and differentiation will address whether EDC exposure negatively impacts their ability to function normally *in vivo* and their potential use in regenerative medicine. Here, human MSCs were used to investigate the mechanism(s) of action of DDT. MSCs were exposed to DDT and the impact on morphology, self-renewal, proliferation, differentiation, and gene expression profiles was examined. The results demonstrate that DDT-treated MSCs exhibit profound alterations in these essential

biological properties, and that these altered processes may reflect homeostatic imbalance and increased cancer incidence among those exposed to EDCs.

Methods

Chemicals

DDT was purchased from AccuStandard (New Haven, CT). 17β-estradiol (E_2), fulvestrant (ICI), dexamethasone, isobuytlmethylxanthine, indomethacin, ascorbate 2-phosphate, β-glycerol phosphate, Oil Red O, Alizarin Red S, cetylpyridinium chloride (CPC), and crystal violet were all purchased from Sigma (St. Louis, MO).

Isolation of MSCs

MSCs from three normal healthy donors (n=3) were obtained after informed consent and under a protocol approved by Tulane University Institutional Review Board. The cells were prepared from bone marrow aspirates using standard protocols as previously described (Strong et al. 2013a). Briefly, bone marrow aspirates were taken from the iliac crest of normal adult donors. Cells were isolated using a density gradient (Ficol-Paque; Amersham Pharmacia Biotech; Milwaukee, WI) and cultured in complete culture media (CCM), composed of α-MEM (GIBCO; Grand Island, NY), 20% fetal bovine serum (FBS; Atlanta Biologicals; Lawrenceville, GA), 100 units per ml penicillin/100 μg/ml streptomycin (P/S; GIBCO), and 2 mM L-glutamine (GIBCO). When the cultures reached 70% confluency, the cells were harvested with 0.25% trypsin/1 mM EDTA, resuspended at $1x10^6$ cells/ml in α-MEM with 5% dimethyl sulfoxide (DMSO) and 30% FBS, frozen in 1 ml aliquots overnight at -80^oC, and stored in liquid nitrogen for no more than six months before thawed. Donor demographic information can be found in Supplemental Material, Table S1.

Cell culture

MSCs (10^6) were thawed, plated in CCM, and incubated at 37°C with 5% humidified CO_2. After 24 hours, viable cells were washed, harvested with 0.25% trypsin/1 mM EDTA, and replated at 100 cells/cm^2 in CCM. Media was changed every 3-4 days. For all experiments, sub-confluent cells (≤70% confluent) between passages 2-6 were used. Where indicated, MSCs from each donor were treated separately with DMSO as a vehicle control, 10 nM estrogen, or 1 μM DDT for 5 days in α-MEM supplemented with 20% charcoal dextrose stripped FBS (CDS-FBS), 2 mM L-glutamine, and P/S. For a dose-response relationship, MSCs were treated with 100 pM, 1 nM DDT, 10 nM DDT, 100 nM DDT, 1 μM DDT or 10 μM DDT. Where indicated, combined treatment of 10 nM estrogen + 100 nM ICI 182,780 or 1 μM DDT + 100 nM ICI 182,780 was administered for 5 days. Images were acquired on Nikon Eclipse TE200 (Melville, NY) with Nikon Digital Camera DXM1200F using Nikon ACT-1 software version 2.7.

MCF7 cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO), supplemented with 10% FBS and P/S. Cells were grown at 37°C with 5% humidified CO2, fed every two to three days, and split 1:4 when they reached 90% confluency.

Colony-forming unit assay

MSCs were plated in triplicate at 1.8 cells/cm^2 in 6-well plates (Nunc) and incubated for 14 days. Plates were washed with PBS, and stained with 3% crystal violet for 30 minutes. Plates were washed and colonies (2 mm^2 or greater in diameter) were counted. Plates were destained with methanol and absorbance values were measured at 584 nm (FLUOstar optima; BMG Labtech Inc.; Durham, NC). Values represent the ratio of the absorbance value to the number of colony

forming units and are shown as arbitrary units (A.U.). Assays were performed three independent times.

MTT assay

Assessment of cell viability was performed according to MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). MSCs were plated in triplicate in 96-well plates (500 cells per well). After 1, 2, 4, and 7 days, cells were incubated with 10 mM MTT (Invitrogen; Grand Island, NY) for 4 hours at 37°C with 5% humidified CO_2. A total of 100 µl of dissolving solution (10% SDS, 0.01 M HCl) was added to each well and incubated for 12-16 hours at 37°C. Absorbance was measured at 544 nm (FLUOstar optima). Each experiment was performed three independent times.

Differentiation protocols

MSCs (10^5) from each donor were plated in 6-well plates in triplicate and cultured in CCM until 70% confluent. For adipogenic differentiation, CCM was replaced with media containing adipogenic supplements, which consisted of 0.5 µM dexamethasone, 0.5 mM isobuytlmethylxanthine, and 50 µM indomethacin. For osteogenic differentiation, CCM was replaced with media containing osteogenic supplements, which consisted of 50 µM ascorbate 2-phosphate, 10 mM β-glycerol phosphate, and 10 nM dexamethasone. After two weeks, cells were fixed, washed, and stained with 0.5% Oil Red O (adipogenic differentiation) or 40 mM Alizarin Red (osteogenic differentiation). Images were acquired on the Nikon Eclipse TE200 with Nikon Digital Camera DXM1200F using Nikon ACT-1 software version 2.7. For quantification, the Oil Red O and Alizarin Red was extracted from each well with isopropanol and 10% CPC, respectively, and read at 584 nm (FLUOstar optima). To normalize to amount of

protein in each sample, protein extraction with RIPA buffer (Pierce; Thermo Scientific; Waltham, MA) and protein quantification with the BCA assay (Thermo Scientific) was performed according to manufacturer's instructions. Experiments were conducted three independent times.

RNA isolation and qRT-PCR

MSCs (10^6) from three different donors and MCF7 cells were treated separately with vehicle, 1 nM estrogen, or 1 μM DDT for 5 days. Total cellular RNA was extracted from MSCs using TRIzol reagent (Invitrogen), purified with DNase I digestion (Invitrogen), and reverse transcribed using the SuperScript VILO cDNA synthesis kit (Invitrogen). PCR was performed in triplicate using the EXPRESS SYBR GreenER qPCR SuperMix Kit (Invitrogen) according to the manufacturer's instructions. Primer sequences for the following factors can be found in Supplemental Material, Table S2. The expression of human β-actin was used to normalize mRNA content.

Sample preparation, cDNA library preparation and next generation sequencing

MSCs (10^6) were treated with 1 μM DDT or vehicle. After five days, cells were trypsinized, washed with PBS, and stored at -80^{o}C prior to RNA isolation. RNA sequencing (RNA-seq) was performed as previously described (Miller et al. 2013). cDNA libraries were prepared from ribo-depleted total RNA and sequenced on an Illumina HiSeq 2000 instrument.

Gene expression and hierarchical clustering analysis

Before RNA-seq reads were aligned to the human genome, three nucleotides from the 5' end of each read were trimmed using PrinSeq [default options]. Reads were then aligned to the human genome (hg19; USCS) using Tophat V2.0.8 [default options] (Trapnell et al. 2009), Transcript

data from Tophat was analyzed using HTseq [-m intersection-strict, default options] (Anders 2010) for transcript quantification of human genes along with non-coding RNAs. The transcript counts were then imported into the R software environment and analyzed using the edgeR package (Robinson et al. 2010). An adjusted $P < 0.05$ following the Benjamini-Hochberg correction was applied for multiple testing for identifying significant differential expression. Significant differentially expressed genes between vehicle and DDT-treated MSCs were clustered using the Pearson and Spearman correlation coefficient and complete linkage algorithms to detect groups of co-expressed genes.

Statistical analysis

All values are presented as means ± standard deviation. Experiments were conducted in triplicates with three independent donors and three separate times for biological replicates. The average of the triplicates with each donor per repeat was averaged together, and the values for each independent trial with respect to each donor were averaged together. Finally, the average of the three donors was averaged together to calculate the mean ± standard deviation. The statistical differences between three or more groups were determined by ANOVA, followed by post-hoc Dunnett multiple comparison tests versus the respective control group. The statistical differences between two groups were performed by Student's t-test. Statistical significant was set at $P < 0.05$. Analysis was performed using Prism (Graphpad Software, San Diego, CA).

Results

DDT alters morphology of MSCs and inhibits self-renewal capacity

To examine the effect of DDT on MSC morphology, cells were treated with DDT in CCM for 5 days. MSCs treated with DDT displayed less fibroblast-like phenotype and more spherical morphology under light microscopy (Figure 1A).

To assess self-renewal capacity, vehicle- and DDT-treated MSCs were plated at low densities. After 14 days, the colonies were counted and represented as the number of colony forming units (CFUs) per plate. The treated MSCs formed 8.1 ± 0.77 CFUs compared to 13.9 ± 1.56 CFUs in the control group, indicating that DDT reduced the self-renewal of MSCs (Figure 1B). The colonies generated by DDT-treated cells were larger than the CFUs from vehicle-treated MSCs, suggesting that those MSCs that were able to self-renew also had greater proliferation potential. The proliferation of DDT-treated MSCs was analyzed by an MTT assay. MSCs exposed to DDT demonstrated increased proliferation by day 7 compared to the control (Figure 1C). To quantitate the CFUs formed by the MSCs, crystal violet was extracted and normalized to the number of CFUs in each sample. Vehicle- and DDT-treated MSCs were on average 0.021 ± 0.0032 A.U. and 0.029 ± 0.0021 A.U. ($P < 0.05$), respectively, indicating that the DDT-treated MSCs produced larger CFUs (Figure 1D). These results indicate that DDT reduces the self-renewal of MSCs, but those MSCs that are able to self-renew have an enhanced proliferation.

DDT enhances the differentiation of MSCs

The differentiation potential of MSCs was evaluated by culturing cells in high-density cultures in either osteogenic or adipogenic induction media. To determine the optimal concentration and the period of treatment, cells were treated with vehicle, 100 nM DDT, or 1 μM DDT for 1 or 5 days.

MSCs treated with 1 μm DDT for 5 days demonstrated the greatest osteogenic and adipogenic differentiation relative to control (Supplemental Material, Figure S1; Figure 2A; Figure 2B). Therefore, all additional MSC studies were performed at 1 μM concentrations for 5 days. MSCs exposed to 1 μM DDT demonstrated a 2.1-fold increase in osteogenic and a 1.8-fold increase in adipogenic differentiation. Furthermore, analysis of osteogenic transcription factors demonstrated an increase in mRNA expression for osteonectin, *CBFA-1*, and *c-Fos* by 2.3-, 2.4-, and 7.5-fold, respectively, compared to control ($P < 0.01$; Figure 2C). Analysis of the expression of adipogenic transcription factors revealed an increase in mRNA expression of leptin, LpL, and PPAR-γ by 2.1-, 10.9-, and 72.3-fold, respectively compared to control, indicating increased adipogenesis ($P < 0.01$; Figure 2C).

DDT impairs the innate stem cell properties

To assess the DDT concentrations that impair MSC function, cells (n=3 donors) were first treated with different concentrations of DDT for five days and then plated for proliferation, CFU, and differentiation assays. DDT acts on MSC proliferation, CFU, and differentiation in a dose-dependent manner. Higher concentrations of DDT resulted in increased proliferation, reduced CFU, and enhanced both osteogenic and adipogenic differentiation in a dose-dependent manner. The EC50 of DDT on proliferation was 102.4 nM (Figure 3A). The IC50 for DDT on self-renewal capacity was 4.2 nM, suggesting that DDT induces robust changes on MSC biology (Figure 3B), and the EC50 of DDT on osteogenic and adipogenic differentiation was 382.2 nM, and 287.7 nM, respectively (Figure 3C). The results also suggest that the effect of DDT plateau at 1 μM. These studies confirm that 1 μM DDT is the optimal dose to elicit a significant response in proliferation, differentiation, and self-renewal of MSCs.

Estrogen receptor antagonist inhibits the effects of estrogen and DDT

The estrogen-like activity of DDT on MSCs was assessed by culturing cells in CDS-FBS and treatment with, ICI182,780, estrogen, DDT or vehicle for 5 days. MSCs were collected on days 1, 2, 4, and 7 and quantified for proliferation. Both estrogen- and DDT-treated MSCs demonstrated enhanced proliferation compared to vehicle-treated MSCs ($P < 0.05$; Figure 4A).

To inhibit the estrogenic activity, ICI 182,780 was simultaneously delivered with estrogen and DDT, and cell proliferation was assessed. The enhanced MSC proliferation induced by estrogen or DDT was blocked by ICI 182,780 (Figure 4B), indicating that the proliferation of MSCs is mediated by ER.

To assess self-renewal potential, MSCs were plated at low densities in CDS-FBS and stained with crystal violet for quantification of CFU after 14 days. MSCs cultured in CDS-FBS treated with estrogen or DDT demonstrated diminished self-renewal capacity, forming 5.3 and 5.6 colonies, respectively ($P < 0.05$). Again, ICI 182,780 treatment inhibited the effect of estrogen or DDT on MSC self-renewal (Figure 4C).

Since MSCs grown in FBS and treated with DDT demonstrated a 1.4-fold increase in the size of colonies, cells cultured in CDS-FBS and treated with estrogen or DDT were also analyzed. CCM was switched to CDS-FBS media to examine the effects of estrogen and DDT, without interference from endogenous estrogen in the serum. Absorbance values for MSCs treated with estrogen and DDT were 0.036 ± 0.0025 A.U. and 0.035 ± 0.0044 A.U., respectively, compared to control cells ($P < 0.05$; Figure 4D). Thus, estrogen and DDT treatment resulted in a 2.1-fold increase in the size of the colonies. The colony size returned to baseline when MSCs were

treated with estrogen + ICI 182,780 (0.018 ± 0.0012 A.U.) or DDT + ICI 182,780 (0.015 ± 0.0003 A.U.), suggesting that ER inhibitors diminished the size of colonies (Figure 4D).

DDT mediates MSC differentiation via an estrogen dependent mechanism

The estrogenic effects of DDT on MSCs were determined by culturing cells in CDS-FBS followed by treatment with estrogen DDT or vehicle. After 5 days of treatment, MSCs were switched to either osteogenic or adipogenic induction media. After 14 days, MSCs were assessed for osteogenic and adipogenic differentiation. MSCs treated with estrogen, DDT or vehicle all demonstrated differentiation along osteogenic and adipogenic lineages (Figure 5A). Upon quantification, MSCs treated with estrogen demonstrated enhanced osteogenic and adipogenic differentiation by 2.2- and 2.4-fold, respectively, and DDT treatment enhanced differentiation by 1.9- and 2.7-fold, respectively (Figure 5B).

To assess the influence of estrogen and DDT on osteogenic and adipogenic factors, treated cells were analyzed by qRT-PCR. The expression levels of key factors involved in osteogenic and adipogenic differentiation increased after exposure to estrogen or DDT ($P < 0.05$; Figure 5C). The mRNA expression of osteogenic factors increased relative to vehicle after treatment: osteonectin increased 6.2- (estrogen) and 6.5-fold (DDT); *CBFA-1* increased 6.0- (estrogen) and 5.2-fold (DDT); *c-Fos* increased 12.7- (estrogen) and 6.3-fold (DDT); osteopontin increased 5.6- (estrogen) and 5.0-fold (DDT); and *DLX5* increased 9.0- (estrogen) and 5.2-fold (DDT) ($P < 0.05$; Figure 5C). Similarly, the expression of adipogenic factors was increased after treatment: *GLUT-4* increased 14.5- (estrogen) and 7.3-fold (DDT), *LpL* increased 2.7- (estrogen-) and 2.6-fold (DDT), *PPAR-Y* increased 2.2- (estrogen) and 2.1-fold (DDT), leptin increased 106.7- (estrogen) and 46.4-fold (DDT); and *FABP4* increased 28.6- (estrogen) and 21.3-fold (DDT) ($P < 0.05$; Figure 5C).

Additional analysis was conducted to determine whether MSCs express ER-α, ER-β, and ER variants, including ER36, ER46, and GRP30, and whether estrogen or DDT simulated the expression of these receptors. MCF7 cells, an ER positive breast cancer cell line, were used as a positive control for the detection of ER and ER variants. MSCs express comparable levels of ER-α, ER46, and GRP30. The addition of estrogen enhanced the expression of ER-α and ER46 in MSCs, while DDT enhanced the expression of ER-α and GRP30 expression (Figure 5D; $P<0.05$). To assess the function of ERs, the mRNA levels of SDF-1 were monitored, as SDF-1 is a downstream target of ER. Increased mRNA expression levels of SDF-1 were observed in MSCs treated with estrogen- and DDT-treated MSCs (Figure 5E; $P<0.05$).

The addition of ICI 182,780 in the presence of estrogen or DDT reduced both osteogenic and adipogenic differentiation of MSCs by 1.5-fold and 2.4-fold, respectively, suggesting that the enhanced differentiation of DDT-treated MSCs is, in part, through the activation of ER signaling (Figure 6).

RNA-seq analysis reveals distinct gene expression profiles for DDT- treated MSCs

To globally investigate the influences of DDT on MSC signaling pathways, the gene expression profiles of MSCs treated with either DDT or vehicle were determined by RNA-seq. A total of 121 genes and non-coding RNAs were differentially expressed at statistically significant levels (Supplemental Material, Figure S2, Table S3, Table S4). Gene clustering revealed 2 separate clusters, and Ingenuity Pathway Analysis software found the two most significantly affected pathways were “cell death and survival, tumor morphology, cancer” and “RNA post-transcriptional modification, cellular assembly and organization, cellular development” (Supplemental Material, Figure S3, Table S3).

Discussion

To our knowledge, this is one of the first studies to examine the effects of DDT on human MSCs. The results demonstrate increased proliferation, diminished self-renewal capacity, and enhanced differentiation in DDT-treated MSCs. Increased expression of mRNAs for several key factors that regulate either adipogenesis or osteogenesis was also demonstrated. Treatment with ICI 182,780 returned MSC pluripotency, proliferation, and self-renewal potential to baseline, indicating that the effect of DDT functions through ER signaling pathways.

Previous studies have focused on the impact of DDT on the reproductive system. However, the results of this study suggest that DDT has negative effects on adult stem cells. As MSCs regulate hematopoietic stem cell development and maturation, alterations in MSC biology may disrupt bone marrow homeostasis. Furthermore, alterations in MSCs have been linked to metabolic diseases and obesity. As MSCs can also give rise to adipocytes, it is of particular interest to determine if exposure to DDT has a direct modulation of lipogenesis, lipolysis, and adipogenesis. It will be important to understand how the set point for adipocyte number is programmed in adults and how alterations by DDT exposure may promote obesity in the adult.

Consistent with previous studies investigating the biological action of DDT in reproductive cells and cancer cells, the results of this study clearly demonstrate an estrogen-like response of DDT in adult MSCs. Estrogen has been shown to influence MSCs by enhancing the proliferative capacity and attenuating the self-renewal capacity (Gregorio et al. 2001; Hong et al. 2011). Estrogen has been shown to diminish self-renewal capacity through the activation of ERα (Gregorio et al. 2001). Furthermore, estrogen enhances osteogenic differentiation through ERα and ERβ, and adipogenesis via the ERα receptor only (Hong et al. 2006; Rajalin et al. 2010). Our results suggest that ERα and ERβ are expressed by ASCs; however, ERα is significantly

simulated by estrogen and DDT. Furthermore, DDT may signal through ER variant GRP30 as its expression is significant enhanced following stimulation by DDT. It should also be noted that while DDT reduced self-renewal capacity, those MSCs that retain the potential to self-renew demonstrate enhanced proliferation. Together, these results suggest that DDT enriches for a subpopulation of MSCs with the potential to self-renew and proliferate rapidly. Our data corroborate DDT-treatment result in changes MSCs, which are similar to those induced by estrogen. Furthermore, the use of ER inhibitors markedly reduced the effects of DDT, providing further support of the activation of DDT through ER signaling.

The global assessment of the gene expression profile of MSCs exposed to DDT demonstrated altered expression of genes involved with cell death and survival, cancer, and cellular assembly and organization. The results from this study provides support for previous population and molecular studies that have indicated increased cancer incidence in children and adults exposed to environmental DDT (Bratton et al. 2012; Cohn et al. 2007; Diel et al. 2002; Guo et al. 2013; Kazantseva et al. 2013). These data indicate that DDT alters MSC biology and suggest that these changes could result in higher cancer incidence in individuals subjected to long-term exposure. Previous studies have shown that alterations in adult stem cells contribute to enhance tumorigenicity and support future studies investigating the interplay between EDCs, adult stem cells and cancer incidence (Strong et al. 2012; Strong et al. 2013b)

Numerous studies have demonstrated that DDT and other chlorinated biphenyl pesticides exert estrogenic activity at both the cellular and molecular levels (Bratton et al. 2012; Diel et al. 2002; Li et al. 2013), but few, if any, have studied the biological changes induced by these chemicals in model systems capable of assessing differentiation outcomes and cell fate such as human MSCs. The use of adult human MSCs provides a powerful biological system to interrogate the

molecular mechanisms underlying the effects of EDCs. In particular, MSCs can be treated with different EDCs and analyzed for self-renewal capacity, proliferative rate, and differentiation into several lineages. While the data presented here describe the altered gene expression profile of MSCs after 5 days of exposure to DDT, additional research is required to determine the impact of long-term DDT exposure on MSC signaling pathways, differentiation, and functionality. Impairment in any aspect of self-renewal or differentiation of the MSCs after treatment with EDCs indicates a significant alteration in the biology of these stem cells and potential cause of increased cancer incidence due to long-term exposure.

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Figure legends

Figure 1. DDT affects MSC morphology, self-renewal capacity, and proliferation. Cells from each donor (n=3) were treated separately with vehicle (DMSO) or 1 μM DDT for 5 days. (A) Representative images are shown. Scale bars represent 200 μm and 50 μm for images taken at 4x and at 20x magnification, respectively. (B) Treated cells were plated at low density. After 14 days, cells were fixed and stained with crystal violet. Colonies larger than 2 mm^2 in diameter were counted. (C) Treated cells were seeded in a 96-well plate and analyzed using the MTT assay on day 1, 2, 4, and 7. (D) Colonies stained with crystal violet were destained and absorbance values were determined. Values are shown as the ratio of absorbance to the number of colonies and represented as arbitrary units (A.U.). Bars, ± SD. *$P < 0.05$.

Figure 2. Enhanced differentiation and transcription factor expression due to DDT in MSCs. MSCs from each donor (n=3) were cultured in CCM and treated with vehicle (DMSO) or 1 μM DDT for 5 days. (A) After the treatment period, the medium was switched to osteogenic or adipogenic differentiation medium. After 14 days, MSCs were fixed and stained with Alizarin Red S for the detection of osteogenic differentiation and Oil Red O for the detection of adipogenic differentiation. A representative image for each group is shown. Original magnification is 4x. (B) To quantify the differentiation after treatment with DDT, stains were eluted and absorbance values were obtained on a plate reader at 584 nm. Samples were normalized to the amount of protein present. (C) qRT-PCR analysis of total cellular RNA prepared from treated cells DDT was analyzed. Changes in the levels of expression of osteogenic and adipogenic factors are shown relative to vehicle-treated MSCs. Scale bars represent 200 μm. Bars, ± SD. *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$.

Figure 3. DDT affects MSC proliferation, self-renewal, and differentiation in a dose-dependent manner. MSCs from each donor (n=3) were treated separately with 10^{-10} M, 10^{-9} M, 10^{-8} M, 10^{-7} M, 10^{-6} M, or 10^{-5} M DDT for 5 days. (A) Treated cells were plated (500 cells/well) in a 96-well plate and analyzed using the MTT assay on day 7 for proliferation. (B) DDT-treated cells were plated at low density. After 14 days, cells were fixed and stained with crystal violet. Colonies larger than 2 mm^2 in diameter were counted. (C) After the treatment period, the medium was changed to osteogenic or adipogenic differentiation medium. After 14 days, MSCs were stained with Alizarin Red S (osteogenic differentiation) or Oil Red O (adipogenic differentiation). Differentiation was quantified by eluting the Alizarin Red S with 10% CPC and the Oil Red O with isopropanol. Absorbance values were obtained following eluting. Samples were normalized to the amount of protein present. Bars, ± SD. *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$.

Figure 4. Estrogen inhibitors reduced proliferation and enhanced self-renewal capacity of DDT-treated MSCs. MSCs from each donor (n=3) were cultured separately in CDS-FBS with vehicle (DMSO), 1 μM ICI, 100nM 182,780, 10nM E_2, 10nM E_2 + 100nM ICI, 1μM DDT, or 1μM DDT + 100nM ICI 182,780 for 5 days. MSCs from each donor were analyzed separately. (A) MSCs treated with vehicle, E_2, or DDT were assessed with the MTT assay for proliferation on day 1, 2, 4, and 7. (B) MSCs treated with vehicle, ICI, E_2, E_2 + ICI, DDT, or DDT + ICI 182,780 were assessed on day 7 for proliferation. (C) Treated MSCs were plated in triplicate at low density (1.8 cells/cm^2) for the assessment of self-renewal capacity and cultured for 14 days followed by staining with crystal violet. Representative plates are shown and colonies greater than 2 mm^2 in diameter were counted. (D) To quantify the extent of the colony formation, crystal violet was eluted from CFUs and absorbance was measured on a plate reader at 584 nm. Values are shown

as a ratio of absorbance to the number of colonies and represented as arbitrary units (A.U.). Bars, ± SD. *$P < 0.05$, **$P < 0.01$.

Figure 5. Estrogen and DDT enhance osteogenic and adipogenic differentiation of MSCs. (A) MSCs from each donor (n=3) were cultured in CDS-FBS with vehicle, 1 μM ICI, 10 nM E_2, or 1 μM DDT for 5 days. The medium was changed to osteogenic or adipogenic differentiation medium. After 14 days, MSCs were fixed and stained with Alizarin Red S for osteogenic differentiation and Oil Red O for adipogenic differentiation. A representative image for each group is shown. Original magnification for bone differentiation images is 4x and for fat differentiation images is 10x. Scale bars represent 200 μm. (B) To quantify the differentiation after treatment with DDT, Alizarin Red S and Oil Red O was extracted and absorbance was measured. Samples were normalized to the amount of protein present. (C) qRT-PCR analysis of total cellular RNA prepared from cells cultured in charcoal dextran stripped fetal bovine serum and treated with vehicle, E_2, or DDT was analyzed. Changes are shown relative to vehicle-treated MSCs. (D, E) qRT-PCR analysis of total cellular RNA prepared from MCF7 cells or MSCs treated with vehicle, E_2, or DDT was analyzed. MCF7 cells were used as positive controls. Bars, ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Figure 6. Estrogen receptor inhibition reverses the effect of DDT on osteogenic and adipogenic differentiation of MSCs. MSCs (n=3 donors) were treated with vehicle, 1 μM ICI, 10 nM E_2, 10 nM E_2 + 100 nM ICI, 1 μM DDT, or 1 μM DDT + 100 nM ICI 182,780 for 5 days. The medium was changed to osteogenic or adipogenic differentiation medium containing CDS-FBS. After 14 days, MSCs were fixed and stained with Alizarin Red S for osteogenic differentiation or Oil Red O for adipogenic differentiation. To quantify the amount of differentiation, stains were eluted

from the plates and read on a plate reader at 584 nm. Samples were normalized to the amount of protein present. Bars, ± SD. *$P < 0.05$.

Figure 1

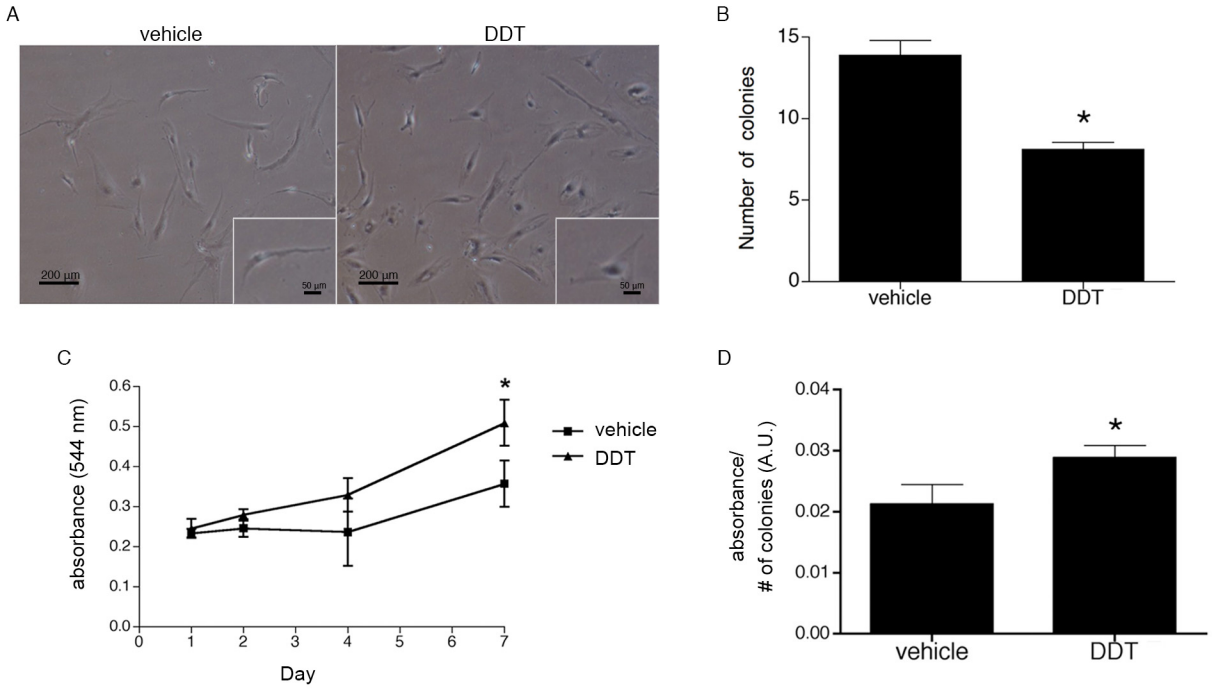

A
vehicle
DDT
200 µm
50 µm
200 µm
50 µm
B
Number of colonies
15
10
5
0
*
vehicle
DDT
C
absorbance (544 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
1
2
3
4
5
6
7
Day
*
vehicle
DDT
D
absorbance/
# of colonies (A.U.)
0.04
0.03
0.02
0.01
0.00
*
vehicle
DDT

Figure 2

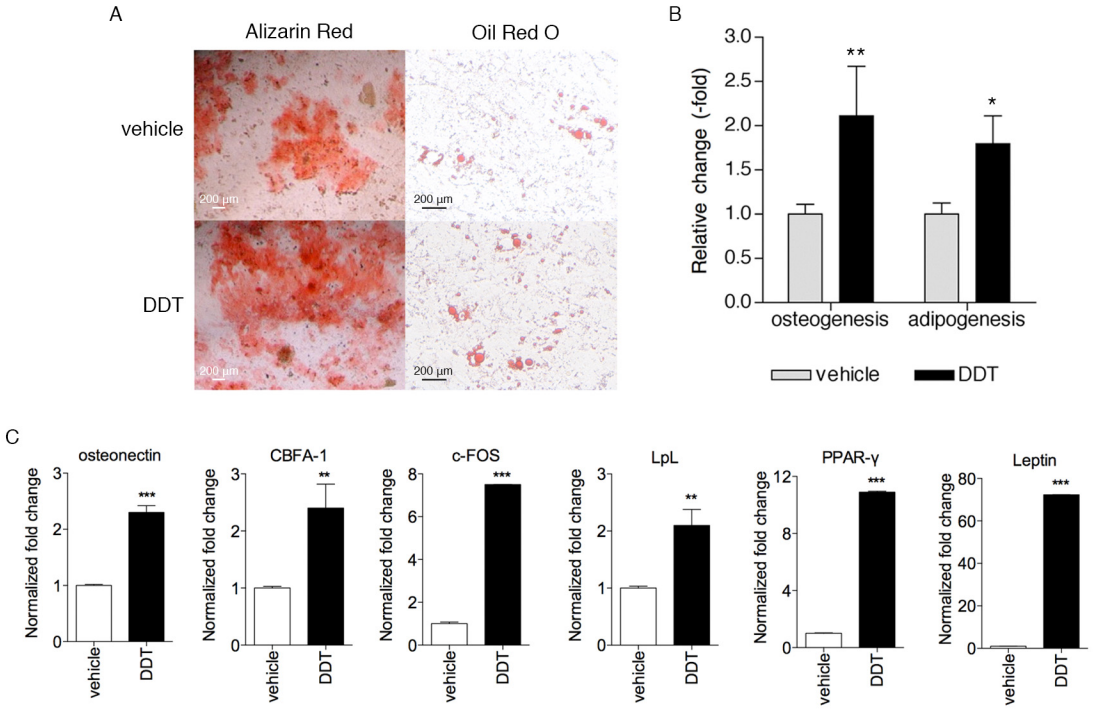

A
Alizarin Red
Oil Red O
vehicle
DDT
200 μm
B
Relative change (-fold)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
*
osteogenesis
adipogenesis
vehicle
DDT
C
osteonectin
CBFA-1
c-FOS
LpL
PPAR-γ
Leptin
Normalized fold change
***
**
vehicle
DDT

Figure 3

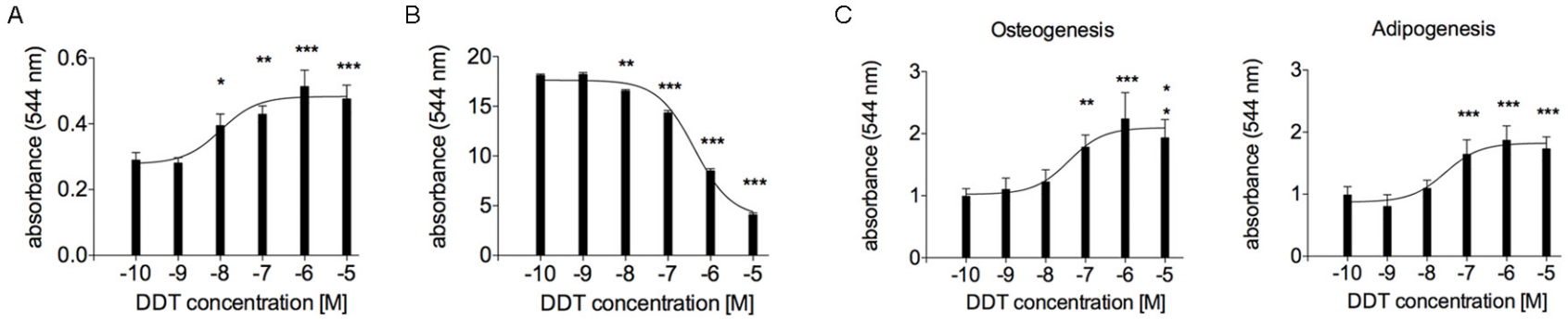
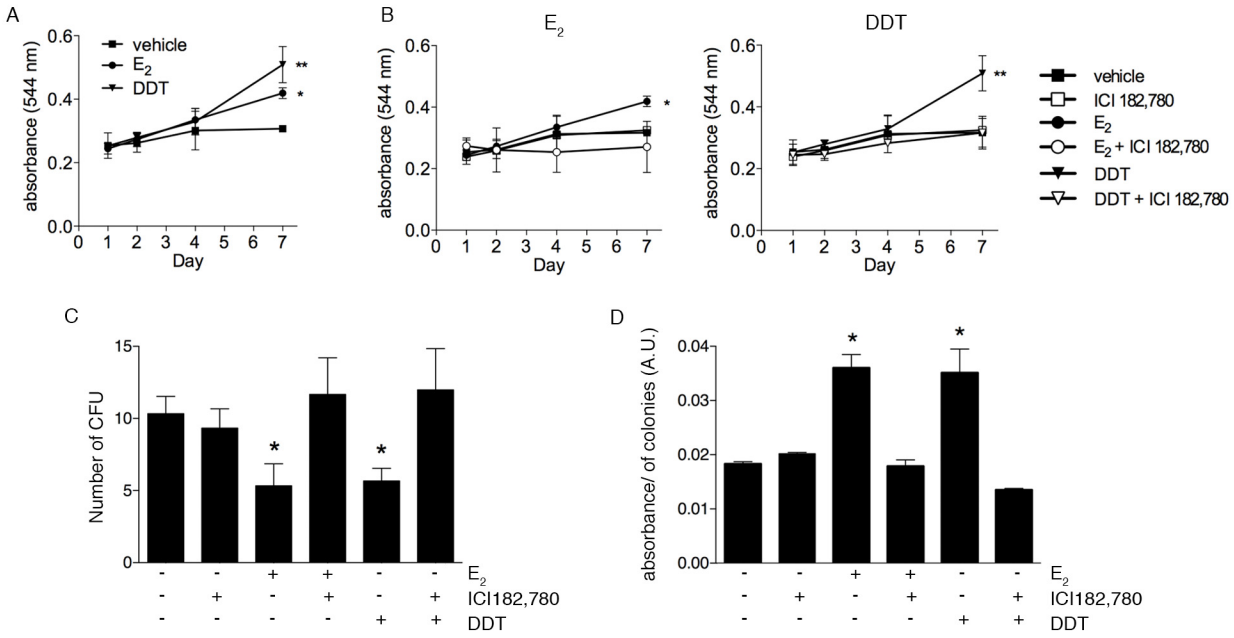

A
absorbance (544 nm)
0.6
0.4
0.2
0.0
*
**
***
***
-10 -9 -8 -7 -6 -5
DDT concentration [M]
B
absorbance (544 nm)
20
15
10
5
0
**
***
***
***
-10 -9 -8 -7 -6 -5
DDT concentration [M]
C
Osteogenesis
absorbance (544 nm)
3
2
1
0
**
***
*
*
-10 -9 -8 -7 -6 -5
DDT concentration [M]
Adipogenesis
absorbance (544 nm)
3
2
1
0
***
***
***
-10 -9 -8 -7 -6 -5
DDT concentration [M]

Figure 4


A
absorbance (544 nm)
vehicle
E2
DDT
Day
B
E2
DDT
vehicle
ICI 182,780
E2 + ICI 182,780
DDT + ICI 182,780
C
Number of CFU
E2
ICI182,780
DDT
D
absorbance/ of colonies (A.U.)

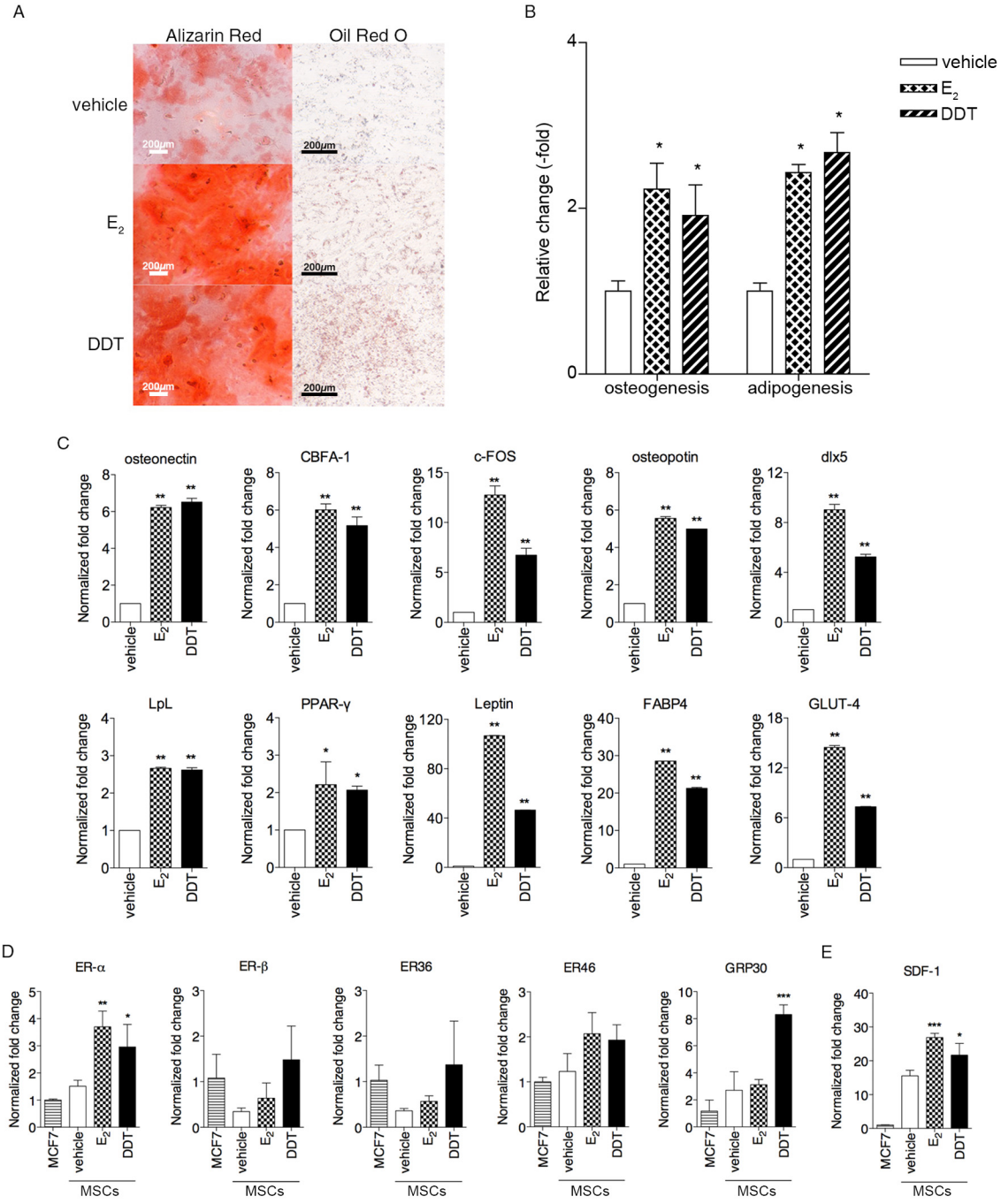
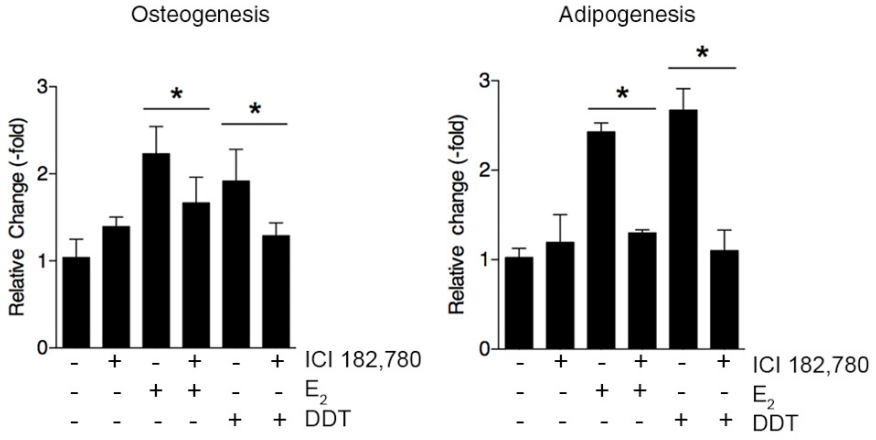

A
Alizarin Red
Oil Red O
vehicle
E2
DDT
200μm
B
Relative change (-fold)
vehicle
E2
DDT
osteogenesis
adipogenesis
C
osteonectin
CBFA-1
c-FOS
osteopotin
dlx5
LpL
PPAR-γ
Leptin
FABP4
GLUT-4
Normalized fold change
vehicle
E2
DDT
D
ER-α
ER-β
ER36
ER46
GRP30
MCF7
vehicle
E2
DDT
MSCs
E
SDF-1

Figure 6


Osteogenesis
Adipogenesis
Relative Change (-fold)
Relative change (-fold)
0
1
2
3
*
*
*
*
- + - + - + ICI 182,780
- - + + - - E2
- - - - + + DDT